SULFATION-INDEPENDENT L-SELECTIN LIGAND (SILL) AND THERAPEUTICS THEREOF

This application is a continuation-in-part application of United States Serial Number 08/321,400 filed October 11, 1994.

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention involves the development of compounds which can regulate and control the function of adhesion molecules as well as methods and apparatus for testing for adhesion molecules.

BACKGROUND ART

Adhesion molecules are involved in the fundamental control of cell-cell interaction and cellular migration. Adhesion molecules regulate diverse processes in inflammation, hematopoiesis and tumor metastasis. (Woodruff, et al, 1987; Springer, et al, 1987; Sharon and Lis, 1993; Sackstein, 1993) For general reviews on adhesion molecules see Carlos and Harlan, 1994 and Chin et al, 1991. It would be

useful to develop reagents which can control and regulate the adhesion proteins, particularly within the selectin family.

The peripheral lymph node "homing receptor", L-selectin (CD62L), is a ~75 kDa glycoprotein which mediates attachment of lymphocytes to lymph node (LN) high endothelial venules (HEV), an adhesive interaction which is the first step in the migration of lymphocytes from blood into lymphoid tissues (Gowans and Knight, 1964; Marchesi and Gowans, 1964). This trafficking of lymphocytes from blood into lymph nodes is markedly nonrandom and is initiated by specific adherence of the lymphocytes to HEV. L-selectin is the principal lymphocyte membrane glycoprotein mediating this attachment (see Shimizu and Shaw, 1993 and also United States Patent 5,489,578 columns 3-4 for a review).

The L-selectin protein is recognized by a variety of monoclonal antibodies (mAbs) in humans (Gatenby et al., 1982 (Leu-8); Reinherz et al., 1982 (TQ-1); Tedder et al., 1990 (LAM)) and is a member of the selectin family of adhesion molecules, which includes P-selectin (CD62P) and E-selectin (CD62E). Selectins share a common structure consisting of an amino-terminal calcium-dependent lectin domain, an epidermal growth factor domain, a variable number of repeat sequences bearing homology to complement

regulatory and catalytic proteins binding C3b or C4b, a transmembrane portion, and a C-terminal cytoplasmic tail (Bevilacqua and Nelson, 1993; Rosen, 1993). The molecular weight varies among leukocytes due to differences in posttranslational glycosylation among subsets of leukocytes (Carlos and Harlan, 1994). The lectin domain of these proteins directs their adhesion to carbohydrate molecules present on the cell surface.

The adhesive interaction between lymphocytes and HEV has been extensively analyzed using an *in vitro* binding assay (Stamper and Woodruff, 1976). The adhesive interaction between L-selectin and its ligand(s) was first identified by this *in vitro* binding assay in which lymphocytes are overlaid onto frozen sections of lymph nodes (Stamper and Woodruff, 1976). This "lymphocyte-HEV adherence assay" is an *in vitro* approximation of physiologic adhesion mediated by L-selectin and has been the conventional approach for studying the adhesive function of L-selectin in its native state on the surface of leukocytes. Indeed, in early studies, antibodies directed against L-selectin were operationally identified by their ability to block lymphocyte-HEV adherence in the assay (Rasmussen et al., 1985; Gallatin et al., 1983), and these antibodies subsequently led to the identification of L-selectin at a molecular level. This assay is performed under shear at 4°C, whereby

binding mediated by L-selectin is maximized and effects of other adhesion molecules are minimized (Shaw et al, 1986; Spertini et al., 1991). Under these conditions, lymphocytes will adhere specifically to lymph node HEV via interactions between L-selectin and its corresponding ligand(s).

The interaction of L-selectin with its corresponding ligand(s) on HEV is calcium-dependent (Woodruff et al., 1977) and requires the presence of sialic acid (Rosen et al., 1985; True et al., 1990) and sulfate (Imai et al., 1993) on the ligand(s) (see United states Patent 5,489,578 column 4 for a review). L-selectin behaves as a lectin and recognizes sialylated, high mannose residues on its corresponding endothelial ligands which are identified by the monoclonal antibody MECA-79 (Streeter, 1988; ATCC Accession number HB-9479; United States Patent 5,403,919).

Most of what is known about L-selectin ligands has been derived from studies of molecules recognized by MECA-79 (Streeter et al, 1988; ATCC Accession Number HB-9479; United States Patent 5,403,919), a rat anti-murine lymph node monoclonal antibody which identifies HEV by attaching to L-selectin ligands. In murine lymph nodes, MECA-79 and a murine L-selectin-Ig chimeric construct each immunoprecipitate several glycoproteins (Watson et

al., 1990), two of which have been well-characterized: GLYCAM-1 (Imai et al., 1991), a secreted protein of 50,000 m.w., and CD34, an integral membrane protein of ~90,000 m.w. (Baumhueter et al., 1993). *In vitro* adherence of lymphocytes via L-selectin can be inhibited by carbohydrates such as mannose-6-phosphate (man-6-P), PPME (Phosphomannan monoester core from *Hansenula hostii*, a phosphomannosyl-rich polysaccharide), and fucoidin (a sulfated, fucose-rich polysaccharide) (Stoolman and Rosen, 1983). In addition, the MECA-79 determinant is found on an HEV glycoprotein of 200,000 m.w. (Hemmerich et al., 1994) and on a selectively modified subset of the mucosal vascular addressin (MAdCAM-1) which supports L-selectin-mediated lymphocyte adhesion under shear (Berg et al., 1993). The HEV ligands recognized by MECA-79 are all heavily glycosylated mucin-like proteins (Shimizu and Shaw, 1993) rich in O-linked glycosylations bearing sialic acid. Treatment of endothelial L-selectin ligands with neuraminidase (Hemmerich et al., 1994; True et al., 1990) and with O-sialoglycoprotease abrogates the binding of L-selectin (Puri et al., 1995).

The molecular determinant recognized by both MECA-79 and L-selectin chimera is sulfation-dependent as demonstrated by markedly diminished ligand recognition following metabolic inhibition of

sulfation by chlorate (Hemmerich et al., 1994). These results have highlighted the critical role of sulfation in ligand activity (Hemmerich et al., 1994; Imai et al., 1993), a conclusion supported by the fact that naturally-occurring unsulfated GLYCAM-1 is not capable of binding L-selectin (Dowbenko et al., 1993). Moreover, sulfation is characteristic of purified glycolipids (Suzuki et al., 1993), oligosaccharides (Greene et al., 1995) and glycosaminoglycans (Stoolman and Rosen, 1983) which bind to L-selectin, and the degree of sulfation correlates with binding affinity (Stoolman and Rosen, 1983).

Although present on endothelial cells in most tissues (Beschorner et al., 1985), CD34 is best known for its expression on the earliest multilineage colony-forming hematopoietic stem cells (Civin et al., 1984). Hematopoietic progenitor cells characteristically express both L-selectin and CD34 (Terstappen et al., 1992), and there is growing evidence that L-selectin plays a role in hematopoiesis (Terstappen et al., 1993; Kobayashi et al., 1994; Dercksen et al, 1995). The characterization of L-selectin and its ligands among progenitor cells is of considerable interest as adhesion proteins regulate cell-cell and cell-stromal interactions fundamental to hematopoiesis.

It would be useful to have strategies which would allow regulation of hematopoiesis since it is regulated by cell-cell and cell-stromal interactions. For example, Terstappen et al (1993) have shown that activation of L-selectin increases the clonogenic capacity of stem cells. Moreover, Dercksen et al (1995) have demonstrated that hematologic recovery following hematopoietic stem cell transplantation in humans correlates with the number of CD34+/L-selectin+ cells infused.

During recovery of immune function following bone marrow transplantation, pathologic changes have been observed following transplantation which interfere with lymphocyte migration and HEV integrity. Further, in addition to changes in lymph node structure, alterations in lymphocyte migration can occur secondary to the effect of pharmacologic agents used in posttransplant therapy such as corticosteroids (Sackstein, 1993). It would be useful to have an agent which can assist in reestablishing lymphocyte trafficking and so immune function following bone marrow transplantation.

The crucial role of adhesion molecules in controlling and directing the inflammatory process indicates that a reagent which interferes with the process, i.e., an anti-adhesive (see for example United States Patents 5,489,578; 5,512,442;

5,304,640), could have anti-inflammatory properties.

Further, cell adhesion molecules are involved in metastasis, therefore it would be useful to develop an anti-adhesive which has anti-metastatic properties. In particular, with the identification of L-selectin on hematopoietic cells, it would be useful to have an anti-adhesive that affects L-selectin in leukemia to decrease the growth and spread of malignant hematopoietic cells throughout the body.

Further, it would be useful to have additional cell markers and monoclonal antibodies directed against these cell markers to allow for cell targeting.

In general, assays for determining the adhesion between cells based on shear require the use of frozen sections of the substrate or target cell (Stamper and Woodruff, 1976; Sackstein et al, 1988) as described hereinabove and a cell suspension overlaying the substrate. This conventional overlay adherence/binding assay has previously been used only with frozen tissue sections, with lymph nodes as a principal example, as the substrate. The frozen section has the advantage of being a predictable monolayer. A monolayer is required since the overlay adherence assay mimics the blood flow over a vessel wall.

It would be useful to have available isolated cells or cell lines as the substrate in the assay. This would enable the use of cell suspensions rather than tissue blocks, giving rise to more reproducible results as well as increasing the types of cells available as a target and reducing the need for surgical procedures for tissue removal.

Initially, applicants attempted to grow the cell lines directly on the glass slides. However, the assay did not produce reproducible results because a predictable, consistent monolayer of cells was not generated. If there is no consistent monolayer, i.e. if there is an uneven surface due to clumping etc. of the cells, then adherence artifacts are found.

Initial attempts to use preparations of single cell suspensions deposited on a glass slide utilizing a Cytospin 3 Cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA) were also generally unsuccessful. Procedures using a cytocentrifuge placed a single layer cell pellet/substrate in the center of the slide (Figure 11, 13, 15). When these slides were used in the assay on a rotating platform (e.g. 80 rpm rotation) in the adherence assay at 4°C for 30 minutes the rotational shear forces placed the overlay cell suspension utilized (typically lymphocytes) at a variety of positions on the slide depending on the platform itself, the platform angle

and the rotation speed as well as other factors. In general, the overlay cell suspension was not localized over the center of the slide above the cytocentrifuged placed cell substrate. It would therefore be useful to be able to modify the placement of the cytocentrifuged cell pellet (to any selected location on the slide) so that it can be used as a substrate in an adherence assay dependent on the shear conditions employed for any given assay.

SUMMARY OF THE INVENTION

According to the present invention, an isolated and purified glycoprotein and functional analogues thereof are disclosed. The glycoproteins are characterized by being expressed on at least primitive hematopoietic cells, and being a ligand for L-selectin. The binding of ligand to L-selectin is sulfation-independent and it is neither inhibited by anti-CD34 antibodies nor by MECA-79 monoclonal antibody and is resistant to O-sialoglycoprotein endopeptidase activity.

Further, the present invention provides a method of performing an overlay adherence assay by using isolated cells or cell lines as a substrate. The cells are prepared as the substrate for the assay using a cytocentrifuge with a modified sample chamber

allowing placement of the cytocentrifuged cell pellet to any selected location on the slide determined by the shear conditions employed for any given assay.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1A-B are photomicrographs of cytocentrifuge preparations of KG1a cells demonstrating adherence of lymphocytes (small dark dots), (**A**) Lymphocytes adhere to KG1a in the presence of CD45 or isotype control antibodies (Abs), (**B**) Lymphocyte binding assay in the presence of LAM1-3 antibody (anti-L-selectin);

FIGURE 2A-C are FACS profiles of lymphocytes used in the binding assay after incubation with (**A**) isotope-matched IgG control, (**B**) LAM1-3, or (**C**) anti-CD45 antibodies, followed by GAM-FITC, results shown are representative of three independent experiments;

FIGURE 3A-D are FACS histogram profiles of KG1a cells sorted by FACS prior to the binding assay into CD34+ (A,B) and CD34- (C,D) fractions using mAb HPCA-2PE, control sorted cell fraction (A,C) were

restained with a FITC-conjugated isotype matched monoclonal antibody, experimental sorted cell fractions were restained for CD34 using mAb QBEND10-FITC with (**B**) positive (>90%) and (**D**) negative (<10%) for CD34, results shown are representative of three independent experiments;

FIGURE 4 is a photomicrograph showing the results of lymphocyte adherence assay performed on the sorted cells, and no differences in lymphocyte adherence were evident among the CD34+ and CD34- populations, adherence to the CD34 negative fraction is shown;

FIGURE 5A-F are FACS profiles of COS-7 cells transfected with either CD34-pCDM8 (E,F) or pCDM8 (mock, **C,D**), then analyzed by FACS and compared to KG1a (**A,B**) for CD34 expression, antibodies used were isotype-matched IgG_1 control (ACE) and anti-CD34 mAb QBEND10 (BDF), lymphocytes did not adhere to CD34- transfected COS-7 cells, despite higher levels of CD34 expression as compared to KG1a cells;

FIGURE 6A-C wherein: (A-B) are FACS profiles of KG1a cells stained with IgM control antibody (A, top) or MECA-79 (B, bottom) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgM and Figure 6 (**C**) is a photograph of a gel showing KG1a and murine lymph node (LN) metabolically radiolabeled with $^{35}SNa_2SO_4$ and immunoprecipitated with MECA-79, murine

anti-human CD43 as a positive control or respective isotype control antibodies;

FIGURE 7A-C are photographs of representative results of adherence assays using suspensions of rat thoracic duct lymphocytes overlaid onto cytocentrifuge preparations of KG1a cells of light microscopy (250×) of lymphocytes (dark circles) adhering to cytocentrifuge preparations of KG1a cells following incubations with RPMI containing buffer alone (A, top), immediately following neuraminidase treatment (B, middle), and neuraminidase treatment followed by twenty-four hour incubation in 10 mM sodium chlorate (C, bottom), note the absence of lymphocyte adherence immediately following neuraminidase treatment;

FIGURE 8A-C are graphs showing representative results of flow cytometric analysis of sialic acid-dependent epitope of CD43 demonstrating recovery of surface CD43 despite inhibition of sulfation by indirect immunofluorescence, wherein (A) KG1a control levels, (B) KG1a immediately following neuraminidase treatment, and (C) KG1a 24 hours following neuraminidase treatment, cells were cultured in the presence of 10 mM sodium chlorate, monoclonal antibody L60 was used as primary antibody and the secondary antibody was PE-conjugated;

FIGURE 9 A,B are photographs of gels wherein (**A**) shows the inhibition of sulfation by sodium chlorate demonstrated by immunoprecipitation/SDS PAGE analysis of ^{35}S-SO_4 incorporation into CD43 and (**B**) is an autoradiograph of SDS-PAGE analysis of total ^{35}S-SO_4-radiolabelled and ^{35}S-methionine/cysteine-d protein in the presence (+) and absence (-) of chlorate, lanes contain equivalent amounts of lysate material obtained from identical numbers of cells cultured in the presence of respective radiolabels for the terminal eight hours of the 24 hour culture period, marked inhibition of sulfation is demonstrated among all sulfated proteins in the presence of chlorate, without significant effects on protein synthesis as shown by equivalent profiles of ^{35}S-methionine/cysteine-radiolabelled proteins, molecular weight markers in kDa are shown at the left of the Figure;

FIGURE 10 is an exploded view of a holder assembly showing a modified sample chamber of the present invention;

FIGURE 11 is a front view of an assembled holder assembly of the prior art;

FIGURE 12 is a front view of an assembled holder assembly of the present invention showing the lateral and vertical offset of the modified sample chamber;

FIGURE 13 is a top plan view of the assembled holder assembly as shown in FIG. 11;

FIGURE 14 is a top plan view of the assembled holder assembly as shown in FIG. 12;

FIGURE 15 is a side view of the assembled holder assembly as shown in FIG. 11;

FIGURE 16 is a side view of the assembled holder assembly as shown in FIG. 12;

FIGURE 17 is a front view of a slide marked for use in the present invention; and

FIGURE 18 is a front view of a holder assembly with a sample chamber of the present invention partially cut away to show cellular deposition at the position indicated on the slide in FIG. 17.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides an isolated and purified glycoprotein that is a sulfation-independent L-selectin ligand (SILL) and functional analogues thereof.

The term analogue as used herein is defined as a SILL variant (alternatively the terms alteration, amino acid sequence alteration, amino acid sequence variant, glycosylation variant can be used) with some differences in their amino acid sequences or

glycosylation pattern as compared to the native sequence of the SILL. Ordinarily, an analogue will be generally at least 70% homologous with the native SILL over any portion that is functionally relevant. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the amino acid sequence of the protein segment of SILL. The homology will extend over a region of contiguous amino acids. The amino acid sequence of an analogue may differ from that of the glycoprotein of the present invention when at least one residue is deleted, inserted or substituted.

Differences in glycosylation may be present between the analogue and the present invention (see United States Patent 5,489,578 column 1, lines 34-67 for a review of carbohydrates in physiologically relevant recognition/adhesion). For glycoproteins, the post-translational sugar modification are, in some cases, the functional determinants of the molecule. The core protein sequence may be important in the spatial display of the sugars but may not contribute to the activity directly. Ordinarily, an analogue will be generally at least 70% homologous with the native SILL over any portion that is functionally relevant. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the glycosylation pattern of the carbohydrate portion

of SILL. The molecular weight of the glycoprotein may vary between the analogue and the present invention due to carbohydrate differences.

Functionally relevant refers to the biological property of the molecule and in this context means an *in vivo* effector or antigenic function or activity that is directly or indirectly performed by a naturally occurring (native) SILL. Effector functions include but are not limited to include receptor binding, any enzymatic activity or enzyme modulatory activity, any carrier binding activity, any hormonal activity, any activity in promoting or inhibiting adhesion of cells to extracellular matrix or cell surface molecules, or any structural role. The antigenic functions essentially mean the possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring SILL. Biologically active SILL analogues share an effector function of the native SILL which may, but need not, in addition possess an antigenic function.

The glycoprotein SILL has the following functional characteristics. It is expressed on at least primitive hematopoietic cells. The glycoprotein is a ligand for L-selectin. The ligand binding to L-selectin is not inhibited by anti-CD34 antibodies and is not recognized by the MECA-79 monoclonal antibody.

The glycoprotein exhibits sulfation-independent function. The glycoprotein maintains L-selectin ligand function following treatment with O-sialoglycoprotein endopeptidase. The glycoprotein is designated hereinafter as sulfation-independent L-selectin ligand, SILL.

Further, SILL, is a membrane associated glycoprotein and functions as an adhesion protein ligand. The glycoprotein mediates attachment of L-selectin expressing cells such as lymphocytes to hematopoietic cells including primitive hematopoietic cells.

SILL was initially identified on KG1a and is similar to endothelial L-selectin ligands in that its activity is calcium-dependent, requires the presence of sialic acid for L-selectin binding, and it is a glycoprotein. However, SILL differs in two fundamental ways from its endothelial counterparts as currently characterized, and represents a novel L-selectin ligand glycoprotein. Firstly, although both mouse and human lymphocytes bind to KG1a through an L-selectin-medicated interaction (Rosen, 1994), neither a murine L-selectin-Ig chimera (LEC-IgG) nor a human L-selectin-IgG chimera attach to KG1a cells, though these molecules recognize endothelial L-selectin ligands (Majdic, 1994).

Secondly, the KG1a L-selectin ligand, SILL, does not contain MECA-79-recognized epitopes. Both flow cytometric analysis of KG1a (SILL) and immunoprecipitation studies of radiolabeled KG1a membrane proteins show no evidence of MECA-79 antigens on the surface of KG1a cells (Figures 6A, 6B), indicating that this epitope is not involved in the binding determinants of the KG1a L-selectin ligand (SILL). Enzymatic cleavage studies and membrane stripping studies under a variety of pH and salt conditions indicate that the L-selectin ligand expressed on KG1a is a non-GPI-linked integral membrane glycoprotein.

Applicant asked whether the ligand of the present invention has the central structural features of other naturally-expressed L-selectin ligands, all of which have been characterized on endothelial cells. The role of sulfation in the binding activity of the KG1a L-selection ligand is at the core of this question, since it was shown in two previous "landmark" reports (Imai et al., 1993; Hemmerich et al., 1994) that the function of endothelial L-selectin ligands is sulfation-dependent. The Hemmerich et al. article in particular describing an L-selectin ligand, emphasized the functional role of sulfation for all endothelial L-selectin ligands, whereas the Imai et al. article focused only on the sulfation-dependent

function of the secreted endothelial L-selectin ligand known as GlyCAM-1. Applicant's new data clearly distinguishes the L-selectin ligand of the present invention in two important ways: (1) it is not recognized by monoclonal antibody MECA-79, an antibody which recognizes the binding domain of all previously identified membrane L-selectin ligands, and (2) it does not require sulfation for binding to L-selectin.

Thus, Applicant has unequivocally established that this ligand is novel - not only in the fact that it is expressed on a hematopoietic cell line but that it is structurally unique from all previously identified endothelial ligands.

Further, there is an independent relationship between P-selectin glycoprotein ligand-1 (PSGL-1) and the SILL ligand activity showing that they are distinct molecules (Example 7).

The present invention also provides for an antibody directed against SILL. The antibodies may be either monoclonal or polyclonal. Murine monoclonal antibodies are initially raised against KG1a cells. The monoclonals that are generated are then screened for the ability to block lymphocyte binding to KG1a. (See also United States Patent 5,130,144 which discloses a method in which a monoclonal antibody is raised against KG1a cells.)

Utilizing these monoclonal antibodies, the SILL is isolated by immunoprecipitation of KG1a membrane lysates as is standard in the art and used for the production of further antibodies as needed. Such methods can be found described Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989, as well as additional methods of isolation and purification as are known in the art.

Additionally, the antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments may also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the protein or peptide fragment of the protein, generally with an adjuvant and, if necessary, coupled to a

carrier; antibodies to the protein are collected from the sera.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The antibody or antibody fragment can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, magnetic

particles, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

The method of targeting cells includes the steps of preparing antibodies directed against SILL as described above and coupling the antibodies to the appropriate agent whether for cell killing, cell selection or cell identification. For cell killing, toxins such as ricin A chain, pseudomonas exotoxin A, diphtheria toxin, other plant and bacterial toxins as well as chemotherapeutic compounds can be coupled in the present invention forming an immunotoxin. For a general review of the antibody-toxin art see Ramakrishnan, 1990.

Cell targeting requires exposing a population of cells to the immunotoxin. A toxin bound antibody can be administered to the appropriate patient and targeted cells killed *in vivo*. The immunotoxin is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to

improved survival rate, more rapid recovery, or improvement or elimination of symptoms.

A pharmacological formulation of the antibody can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants of the targeted monoclonal antibodies, or iontophoretic, polymer matrices, liposomes, and microspheres can be employed. Examples of delivery systems useful in the present invention include: U.S. Patent Numbers 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

Alternatively, cells can be removed from the patient and treated *ex vivo* selectively. For example, cells expressing SILL can be removed through complement-mediated lysis from the *ex vivo* population and the remaining cells returned to the patient. Additional cell removal can be undertaken utilizing cell sorting, "panning" and magnetic bead separation. Alternatively, utilizing cell sorting, "panning", magnetic bead separation and the like cell populations

can be enriched for SILL bearing cells and this enriched cell population returned to the patient if needed.

The targeted cells to be removed are cells expressing SILL and can be selected from the group consisting of leukemic cells, malignant hemopoietic progenitor cells, or any malignant cell expressing the marker.

The present invention also provides a method of regulating hematopoiesis, particularly in reconstitution of the immune system following bone marrow transplantation. The present invention includes the steps of selecting those cells with high(+) or low(-) expression of SILL depending on the growth characteristics associated with the marker density needed by the patient. The selection procedure utilizes *ex vivo* methods as described herein. After selection, the selected cell type is cultured *in vitro*, if needed, to expand the population using standard methods known in the art. The patient is then infused with the expanded, enriched SILL+ or SILL- population as needed.

The present invention further provides a method of regulating inflammatory response by interrupting cellular migration into lymph nodes and sites of both acute and chronic inflammation including the step of administering to the patient either

functional analogues or antibody directed against SILL thereby disturbing cellular migration mediated by SILL by blocking the L-selectin-mediated cell attachment site and can be injected directly at the inflamed site if needed. The regulation of the inflammatory response would be useful in autoimmune disorders, post-ischemic tissue injury and sepsis (Carlos and Harlan, 1994). Administration and effective dose are as described for immunotoxins hereinabove.

See also United State Patents 5,484,891; 5,489,578; 5,464,778; 5,304,640; 5,538,724 and 5,403,919 for further methods and discussion of treatments with selectin ligands, all incorporated by reference in their entirety.

As described herein the adhesive interaction between cells is analyzed using a modified *in vitro* binding assay (Stamper and Woodruff, 1976). The *in vitro* binding assay requires that a cellular suspension be overlaid onto frozen tissue sections (Stamper and Woodruff, 1976). The assay requires that the substrate be positioned such that the forces of the rotating platform will deposit the overlay cell suspension on top of the substrate.

The present invention provides for the use of isolated cells instead of frozen tissue sections as the substrate of the binding assay. The cells may be established cell lines or isolated cells from any

source, mature or immature, including peripheral blood samples, from bone marrow aspirates, and from tissues donated through organ harvests or other sources as appropriate.

To prepare the cell substrate a protocol is used as further described in Example 1, which requires a modification of an existing sample chamber for use in a cytocentrifuge. When the prior art sample chamber as shown in Figures 11, 13 and 15 is used the cell substrate pellet from the cytocentrifuge is deposited in the middle of the slide to be used as the substrate. As described herein above this is not the preferred location for an overlay adherence assay. The preferred location must be determined for each rotation table and the substrate deposited at that site.

United States Patents 4,391,710, 4,678,579 and 4,729,778 disclose the cytocentrifuge and holder assembly of the prior art. The design and structure of a cytocentrifuge and the general design and construction of the holder assembly and sample chamber are disclosed in these patents and are incorporated in their entirety by reference. The terms used in describing a preferred embodiment of a modified sample chamber of the present invention are the same as in these patents where possible.

The present invention allows the use of isolated cells or cell lines as a substrate, the cells being able to be deposited on a selected location of a slide which must be determined for the shear conditions employed for any given adherence assay as described herein above. The present invention provides a sample chamber assembly comprising a sample chamber including cell substrate receiving means for receiving a cell substrate and depositing means for placing the cell substrate on a slide surface during cytocentrifugation, connecting means for connecting together a slide and sample chamber and fixing the depositing means at one of a plurality of positions relative to the connecting means. The present invention further provides a method of making a sample chamber assembly by connecting together a slide and a sample chamber, the sample chamber including a cell substrate depositing port and fixing the cell substrate depositing port at one of a plurality of positions relative to the slide.

In an embodiment, a sample chamber, generally shown at 20, is shown in Figure 10 which is an exploded view of a sample chamber assembly, generally shown at 22. The sample chamber assembly 22 includes a holding member generally shown at 24, a slide 26, a filter card 28 and the sample chamber 20. The holding member 24 is also referred to as a slide

clip.

The sample chamber 20 generally comprises a flat, generally rectangular plate 30 having a rectangular end flange 32 disposed normal to and along an edge of the plate 30 such that the intersection of the rectangular plate divides the end flange into two side areas, 33,33'. The rectangular end flange 32 and plate 30 are reinforced and interconnected by a generally triangular upper end piece 34 as best seen in Figure 10. In the prior art as shown in Figures 11 and 15 there is a lower end piece 36 opposite and spaced from the upper end piece 34, which is optionally present in the present invention. A vertical directed funnel 38 is contained within the plate 30 and interconnects with a horizontally extending discharge port 40 which extends from the funnel 38 within the plate 30 to the end flange 32. The discharge port 40 terminates at an opening 42 in the end flange 32. In the prior art sample chamber the distance between the opening 42 of the discharge port 40 and each of opposite long sides 44, 44' of the rectangular end flange 32 is equidistant. In other words in the prior art the two side areas 33,33' are generally the same size.

As described in the 4,391,710, 4,678,579 and 4,729,778 patents cell suspensions are loaded in the funnel 38 and deposited on the surface of the slide 26

opposite the opening 42 of the discharge port 40.

A filter card 28 is disposed between the end flange 32 and the slide 26. The filter card 28 is dimensioned to be coextensive with the end flange 32 and is provided with an opening 46 that is coextensive with the opening 42 of the discharge port 40. In an embodiment the filter card 28 may have two openings 42 positioned such that the filter card 28 may be disposed between the end flange 32 and slide 26 in either vertical orientation such that the filter card opening 46 will be coextensive with the opening 42 of the discharge port 40. During operation the filter card 28 will absorb the liquid content of the cell sample and the cells will be deposited on the slide 26. The filter card 28 can be separate as shown in Figure 10 or can be formed as part of the sample chamber 20 as described in United States Patent 4,678,579.

In the present invention the end flange 32 includes opposite lateral edge regions 48, 48'. The lateral edge regions 48, 48' are disposed along each of the long sides 44, 44' of the rectangular end flange 32. In the present invention one of the lateral edge regions 48 is removed as best shown in Figures 12, 14 and 18. The removal of one of the lateral edge regions 48 allows the lateral displacement or offset of the sample chamber 20 in the

sample chamber assembly 22 as best shown in Figure 12, 14 and 18. To allow removal of one of the lateral edge region 48 the lower end piece 36 may have to be removed. The lateral displacement is in the direction of the removed lateral edge region 48 removed. Further the amount of one of the lateral edge region 48 removed is generally such that the distance from the opening 42 of the discharge port 40 in the end flange 32 to the resulting long side 44 of the end flange 32 after removal of the lateral edge region 48 generally corresponds to A as shown in Figure 17 as will be described and is no longer equidistant to the opposite long side 44' of the rectangular end flange 32. In other words the two side areas 33,33' are no longer the same size, and in this embodiment 33 is smaller than 33'. Alternatively, the lateral edge region 48' can be removed. The filter card 28 is modified to be co-extensive with the end flange 32 after one of the lateral edge regions 48, 48' is removed.

The holding member 24 includes a generally U-shaped clip 50 which pivots over the slide 26, filter card 28 and sample chamber 20 when they are positioned in the holding member 24 to hold them in position in the holding member 24. The front surface of the end flange 32 is provided with horizontally directed, slotted ledges 52 on opposite sides of the

discharge port 40. The clip 50 when locked into position to hold the sample chamber assembly 22 together is disposed on the ledges 52 in the prior art as best shown in Figures 11 and 15. In the present invention when the sample chamber 20 is moved laterally, the clip must be disposed on both sides of the discharge port 40 and generally on the ledges 52 as best shown in Figure 12.

In the present invention the end flange 32 and rectangular plate 30 include a coextensive lower edge region 54. The lower edge region 54 is disposed along the lower short side of the rectangular end flange 32 and disposed along the lower edge of the rectangular plate 30 and spaced and parallel to the discharge port 40. In an embodiment of the present invention the lower edge region 54 is removed as best shown in Figures 16 and 18. The removal of the lower edge region 54 allows the vertical displacement or offset of the sample chamber 20 in the sample chamber assembly 22 as best shown in Figure 16. Further the amount of the lower edge region 54 removed is generally such that the distance from the center of the discharge port opening 42 to the resulting lower edge after removal of one of the lower edge region 54 generally corresponds to A' as shown in Figure 17 as will be described.

In the present invention when the sample chamber 20 is displaced vertically, the clip 50 while disposed on both sides of the discharge port 40 will generally not be disposed on the ledges 52 as best shown in Figure 16 but directly on the front surface of the end flange 32.

In an embodiment one of a lateral edge region 48, 48' and a lower edge region 54 are removed allowing a combined vertical and lateral displacement of the sample chamber 20 as best shown for one embodiment in Figure 18.

The modified sample chamber 20 can be made either by adapting existing prior art sample chambers formed in a plastic or other material which can be cut or shaved to size or the modified sample chamber 20 can be formed utilizing the methods disclosed in United States Patents 4,391,710, 4,678,579 and 4,729,778 and methods known to those skilled in the art of plastic extrusion or forming in other materials. A series of modified sample chambers can be prepared having incremental changes in the amount of lateral edge regions 48, 48', which opposite lateral edge region 48, 48' is elected for modification and/or lower edge region 54 removed allowing for different A and A' distances and prepared as a kit. The incrementally modified chambers are then available for testing to determine which

incremental change will deposit the cell substrate at the proper position on the slide.

Alternatively, the holding member 24 can be adapted to allow displacement of the slide 26. The holding member 24 is comprised of a channel 58, which can be U-shaped or M-shaped, having opposite side plates 60, a common web 62 interconnecting the opposite side plates 60 and a pivot rod 66 for interconnecting to the centrifuge. A retaining hook 64 is disposed on each side plate 60 to retain the clip 50 in the locked position. To adapt the holding member 24, one of the side plates 60 can be bent outwardly such that the slide 26 can be displaced laterally while the sample chamber 20 is either not moved or is moved oppositely laterally and/or vertically. This allows for maximum freedom of positioning of the cell pellet 56 on the slide 26. In adapting the side plate 60, care must be taken to insure that the retaining hook 64 can still be used.

The depositing means include the discharge port 40 and the discharge port opening 42 through which the cell substrate pellet 46 is deposited on the slide 26 at one of a plurality of positions. The depositing means can further include the filter card 28 and filter card opening 46 which is coextensive with the discharge port opening 42.

The receiving means include the funnel 38 interconnected with the discharge port 40 such that under cytocentrifugation a cell substrate 56 disposed in the funnel 38 enters the discharge port 40.

The connecting means comprise the holding member 24, end flange 32 and rectangular plate 30 as well as the lateral edge regions 48,48', side areas 33,33' of the end flange 32 and side plates 60 of the holding member 24.

The modified sample chamber 20 (and/or holding member 24) allows for the deposition of a cell substrate 56 at a specified reproducible location on a slide. The location is chosen such that the cell pellet 56 deposited by the modified sample chamber 20 can function as a substrate in an overlay assay.

The determination of the point on the slide on which to deposit the cell substrate is determined empirically as it will differ slightly for each individual rotating platform (rotating table, for example a Labline Orbit Shaker) due to mechanical differences as well as for the cell types, cell density, slide angle, rotational speed, temperature and other factors known to those skilled in the art. The determination can be made by depositing a test cell suspension on a slide that is on the rotating platform. The slide is then observed under the adherence assay test conditions and the area where the

cells aggregate is identified. The distances A and A' and the distance B from the center of the slide is determined for the area of cell aggregation as shown in Figure 17. A sample chamber 20 is then modified as described herein above to deposit the cytocentrifuge cell pellet substrate 56 at the calculated position as shown in Figure 18. Alternatively, a series of incrementally modified sample chambers 20 can be used to establish test slides and the modified sample chamber 20 that provides a cytocentrifuge cell pellet substrate 56 with the most appropriate positioning under the shear conditions established for the given adherence assay can be used. In Example 1 a protocol for an assay to test L-selectin ligand adhesion interactions is described, however other adherence assays requiring shear forces can also be undertaken with the present invention.

Selectins are involved in a variety of adhesive interactions, with biological roles ranging from acute inflammation (regulation of leukocyte trafficking) to immune responses (activation of T cells and regulation of lymphocyte migration to lymphoid tissues) to hematopoiesis. Studies by applicant on the interaction of L-selectin and ligands on hematopoietic cells which function as an adhesive receptor-ligand pair, i.e. *in vitro* binding studies of lymphocytes to KG1a, a primitive CD34-positive human

cell line derived from an acute myeloid leukemia (Civin et al., 1994; Koeffler et al., 1980) lead to the present invention. These studies surprisingly revealed highly specific adherence of lymphocytes to KG1a cells mediated by L-selectin on the lymphocyte, but unexpectedly not involving CD34 as the corresponding ligand as had been previously reported (Baumhueter et al, 1993). The results indicated the presence of a ligand, designated SILL (the ligand has also been referred to as hematopoietic cell L-selectin ligand, HLL), for L-selectin on the surface of this hematopoietic progenitor cell line and provide the first evidence of sulfation-independent L-selectin-mediated adhesion between lymphocytes and a non-endothelial cell type.

For these studies, the lymphocyte-HEV adherence assay was used which is an *in vitro* approximation of physiologic adhesion mediated by L-selectin was used and as discussed herein above is the standard assay for determining adhesion. This assay required the functional interaction of respective membrane structures localized within natural cell ligand bilayers. It has been a fundamental tool in studying the function of L-selectin in its native state on the surface of lymphocytes. This binding assay was novelly adapted as described herein (Figures 10-18) to examine lymphocyte-hematopoietic cell

adhesion, and the results provide the unexpected results of L-selectin-dependent adhesive interactions between lymphocytes and non-endothelial, hematopoietic cells.

Blood cell formation depends critically upon discrete cell-cell and cell-matrix adhesive receptor/ligand interactions which create specialized bone marrow microenvironments wherein cells proliferate and mature. L-selectin is characteristically expressed on a subpopulation of bone marrow cells which includes the earliest multilineage hematopoietic stem cells. Evidence that L-selectin may function in hematopoiesis has been obtained from both *in vitro* clonogenic assays (Koenig et al., 1994) and from clinical engraftment data following bone marrow transplantation (Dercksen et al., 1995).

L-selectin ligands have been recognized heretofore only on endothelial cells and the function of all have been shown to be sulfation dependent (see United Stated Patent 5,489,578). The detection of a sulfation-independent L-selectin ligand on a non-endothelial cell (Example 6) expands the physiologic implications of L-selectin function beyond its well-characterized role in regulating leukocyte trafficking.

The adaptation of the assay allowed for the first time the use of cell lines in a lymphocyte-HEV adherence assay. In this assay, slides were prepared utilizing a modified cytocentrifugation apparatus (Figures 10-18) of KG1a cell suspensions which were used in place of slides of frozen lymph node sections as taught by the prior art. The KG1a cells were placed on the slides by using cytocentrifugation as further described hereinbelow in Example 1.

Several independent lines of evidence indicate that lymphocyte binding to KG1a is mediated primarily, if not solely, by L-selectin. First, an anti-L-selectin mAb (LAM1-3) previously shown to block L-selectin-mediated adherence to LN HEV (Spertini et al., 1991), completely inhibited PBL from binding to KG1a or LN HEV, whereas anti-CD45 and isotype control antibodies did not block lymphocyte binding. Second, L-selectin-mediated binding is a calcium-dependent event, and lymphocytes were unable to bind to KG1a in the presence of the calcium chelator EDTA. Third, carbohydrates such as man-6-P, PPME, and fucoidin inhibited lymphocyte adherence to KG1a. These compounds are all known to bind to L-selectin and to inhibit lymphocyte binding to HEV in the *in vitro* assay (Stoolman and Rosen, 1983; Stoolman et al., 1984). Lastly, it is known that PMA treatment of lymphocytes causes shedding of membrane L-selectin via

a protein kinase C activation pathway, and corresponds to the loss of lymphocyte binding to LN HEV in the *in vitro* assay (Tedder et al., 1990). In these studies, PMA-treated PBL were no longer able to bind to KG1a.

These results revealed that KG1a possess a high affinity L-selectin ligand, however, a significant role for CD34 in this ligand activity was excluded by the following observations: (1) treatment of KG1a cells with a variety of anti-CD34 monoclonal antibodies, singly and in combination, had no effect on binding; (2) the CD34-positive human hematopoietic cell lines RMPI 8402 and MO7e did not support lymphocyte adherence despite higher levels of CD34 expression than that of KG1a; and (3) KG1a cells separated into subpopulations expressing high and low levels of CD34 showed equivalent binding (Oxley and Sackstein, 1994b). Treatment of KG1a with the enzymes neuraminidase, chymotrypsin, and bromelain abrogated lymphocyte binding to the cells, indicating that the ligand is a glycoprotein and that expression of ligand sialic acid is critical for function (Oxley and Sackstein, 1994b).

The nature of the ligand was investigated by determining the effects of various enzyme treatments of KG1a on the binding capacity. Previous studies have shown that ligand expression of sialic acid is essential for L-selectin-mediated binding of

lymphocytes to LN HEV (Rosen et al., 1985). In the present examples, neuraminidase-treated KG1a showed a complete loss of lymphocyte binding, indicating that sialic acid residues are also a necessary component on the KG1a L-selectin ligand; as such, lymphocyte adherence to KG1a involves carbohydrate motifs and is not based strictly on protein-protein interactions. This finding, combined with the results of protease experiments, indicates that the KG1a ligand is a glycoprotein.

To examine whether O-linked glycosylations on the ligand play a central role in the adhesive interaction, KG1a were digested with the enzyme O-sialoglycoprotein endopeptidase which specifically cleaves proteins at sites of O-linked sialo-glycosylation (Abdullah et al., 1992) and which has been shown to differentially cleave epitopes of the CD34 molecule (Sutherland et al., 1992). The data revealed that treatment of KG1a in suspension with the enzyme actively destroyed CD34 epitopes, yet had no effect on lymphocyte adherence. These results suggest that ligand sialic acid residues critical to binding are present on N-linked rather than on O-linked glycosylations.

The functional dependence on intact sialic acid moieties is consistent with results from endothelial L-selectin ligands (True et al., 1990) and

suggested that epitopes critical for binding might be structurally conserved among these ligands. However, treatment of KG1a with O-sialoglycoprotease resulted in characteristic loss of enzyme-sensitive epitopes from CD34 (Sutherland et al., 1992) but did not affect adherence (Oxley and Sackstein, 1994b). Since the ligand activity of endothelial CD34 is eliminated by this treatment (Puri et al., 1995), this result provided further evidence that the KG1a ligand activity was not attributable to CD34, and, more generally, that the binding determinant of the ligand differs structurally from proteins recognized by MECA-79.

CD34 has been reported to be a ligand for L-selectin based on the finding that a murine L-selectin -IgG chimera molecule precipitated CD34 from a murine lymph node lysate (Baumhueter et al., 1993). The results as set forth in the examples indicate that CD34 as expressed on KG1a is not a functional ligand for lymphocyte L-selectin, as no difference in lymphocyte binding to sorted CD34- and CD34+ KG1a cells was observed (see Figures 3 and 4). Titration studies using varying proportions of KG1a and HL60 have demonstrated that the amount of lymphocyte adherence is directly proportional to the percentage of input KG1a cells, indicating that differences in lymphocyte binding to the positive and negative sorted

fractions would have been evident if CD34 were the ligand. It is unlikely that a particular binding epitope of CD34 as selected, as this experiment was done using two different anti-CD34 mAbs to sort the KG1a. Two forms of CD34 on KG1a have been reported ("truncated" and "full length") (Krause et al., 1993); however, these differences do not account for the data herein as sorting was also performed using QBEND10, which recognizes both forms.

In addition to sorting experiments, evidence that CD34 is not the L-selectin ligand on KG1a is derived from mAb blocking studies and adherence assays using other CD34 positive cells. None of the anti-CD34 mAbs tested, or any combination thereof, was able to block lymphocyte binding to KG1a. Furthermore, lymphocytes did not adhere to another primitive CD34+ cell line, RPMI 8402, and transfection of CD34 into COS-7 cells did not confer lymphocyte binding capacity. While potential glycosylation differences of the CD34 molecule expressed by these cell types could affect their ability to support lymphocyte adherence, this explanation is unlikely in light of equivalent adherence observed among the sorted CD34+ and CD34- KG1a cells. Taken together, the data presented here indicate that the CD34 glycoform present on hematopoietic cells is not a ligand for L-selectin. Moreover, flow cytometric analysis of the

various cell lines utilized in the binding assay provides evidence that membrane structures such as LFA-1, VLA-4, CD44, Sialyl Le^x and CD43 do not play a primary role in lymphocyte adherence to KG1a since each of these molecules were also present on at least one other cell line tested that did not demonstrate lymphocyte binding.

SILL is not recognized by MECA-79 monoclonal antibody (Example 5) which identifies L-selectin ligands on lymph node HEV. Immunofluorescence analysis of KG1a using MECA-79 shows no evidence of the protein identified by MECA-79. SILL is shown to be unique from L-selectin ligands thus far identified.

The relationship of the KG1a ligand to proteins identified by MECA-79 were evaluated by performing adherence studies in the presence of MECA-79, flow cytometric analysis of MECA-79 antigens on KG1a, and immunoprecipitation studies of metabolically radiolabeled KG1a and lymph node organ cultures. Incubation of KG1a with MECA-79 did not interfere with lymphocyte adherence in the binding assay and flow cytometric studies showed no evidence of MECA-79 antigen on KG1a (Figure 6A-B). Moreover, no MECA-79-immunoprecipitable proteins were detected from lysates of metabolically radiolabeled KG1a cells using either $^{35}SO_4$ (Figure 6C) or ^{35}S-methionine/cysteine, though characteristic bands were obtained from respectively

radiolabeled murine lymph nodes (Figure 6C). The absence of MECA-79-precipitable protein within KG1a lysates indicates that there are no intracellular accumulations of MECA-79 antigen, and, furthermore, that the MECA-79 antigen is not synthesized by the KG1a cells.

Direct cell-cell interactions were utilized to detect the presence of an L-selectin ligand on a hematopoietic cell. Other studies directed at identifying L-selectin ligands have relied on molecular approaches utilizing a murine L-selectin-IgG chimera molecule, synthesized in a human embryonal kidney cell line, as a probe (Watson et al., 1990). Of note, studies utilizing this chimera have failed to demonstrate binding of the molecule to KG1a cells (Majdic et al., 1994). In general, tissue- and species-specific patterns of glycosylations are well described, (Yamashita et al., 1983; Cullen et al., 1981; Yamashita et al., 1985) and such differences can affect the biological activity of proteins expressed in different cells (Cowing 1983; Huff et al., 1983). As it is known that glycosylation of L-selectin varies among different cells expressing the protein (Lewinsohn et al., 1987, Ord et al., 1990; Griffin et al., 1990), such differences may account for the observation here that native L-selectin, expressed on lymphocyte membranes, selectively binds to a

corresponding ligand on KG1a cells while the chimera apparently does not. Similarly, differences in glycosylation of CD34 among endothelial cells and hematopoietic cells may account for the differential capacity of this protein to participate in L-selectin interactions among these cell types.

Recognition of endothelial ligands by MECA-79 is sulfation-dependent, however, absence of MECA-79 reactivity does not in itself indicate that sulfation does not participate in the binding determinant of the KG1a ligand of the present invention. Applicant directly examined this by measuring ligand activity following incubation with chlorate (Example 6), a highly efficient metabolic inhibitor of both saccharide and peptide sulfation (Baeuerle and Huttner, 1986). The results of chlorate incubation on L-selectin ligand activity of KG1a was evaluated and the results are shown herein below (Table 3 and Figure 9). Incubation of KG1a in chlorate-containing media was performed following complete desialylation of the cells by neuraminidase treatment. Initial studies of the kinetics of recovery of binding activity following either neuraminidase treatment or protease digestion of membrane protein showed absence of ligand activity for twelve hours; thereafter, ligand activity increased in a linear fashion with return to baseline levels within twenty-four hours. Return of binding

activity following desialylation or protease treatment was blocked by metabolic inhibition of N-linked glycosylation by tunicamycin treatment, suggesting that re-expression of activity results predominantly from synthesis/processing of nascent ligand and not from transport to membrane of stored mature protein (Table 3).

Accordingly, KG1a were incubated in chlorate for twenty-four hours to inhibit sulfation throughout the duration of ligand reexpression. $^{35}SO_4$-pulse radiolabeling studies at eight hour time intervals within the twenty-four hours of chlorate incubation indicated that sulfation was inhibited throughout the entire incubation period, as demonstrated by trichloroacetic acid-precipitable radiolabeled protein counts (chlorate-incubated counts consistently <10% that of non-chlorate treated controls) and SDS-PAGE analysis of radiolabeled protein of cell lysates. As shown in Table 3, the binding activity of the KG1a ligand was unaffected by chlorate incubation, though inhibition of sulfation was confirmed, even for the terminal six hours of the incubation period, by a marked reduction of $^{35}SO_4$ incorporation into CD43, a sulfated sialomucin (Wilson and Rider, 1992) expressed on KG1a (Oxley and Sackstein, 1994b) (see Figure 9). The data shows that sulfation, regardless of whether it may be present on protein or sugar, is not critical

to the functional interaction with L-selectin. These data, together with the lack of MECA-79 reactivity, indicate that sulfation does not contribute significantly to the function of the KG1a ligand.

The data presented herein expands the understanding of the structure of selectin ligands and provides the first evidence of a functional, membrane glycoprotein L-selectin ligand whose binding activity is not sulfate-dependent. Multiple studies have emphasized the importance of sulfation in the binding determinant of ligands for both L-selectin (Hemmerich et al., 1994; Imai et al., 1993; Suzuki et al., 1993; Green et al., 1995; Stoolman and Rosen, 1983; Green et al., 1992; Hemmerich and Rosen, 1994b) and P-selectin (Aruffo et al., 1991; Sako et al., 1995; Pouyani et al., 1995), and all previously identified naturally-occurring membrane ligands for these selectins bear sulfate-dependent activity. The contribution of sulfate modifications may relate to the localization of negative ions, on either sugar (Hemmerich and Rosen, 1994b) or protein (Sako et al., 1995; Pouyani, 1995; Niehrs et al., 1992) determinants. Such a role for charge is supported by the finding that unsulfated anionic polysaccharides such as polymers of phosphated mannose can bind to L-selectin (Yednock et al., 1987). Within the KG1a ligand, it is possible that glycosidic and/or amino acid modifications such as

hosphorylation, or the molecular composition of the discrete sugars or amino acids comprising the binding domain, create a relevant anionic milieu. Although the precise structural features which direct binding activity for this and other L-selectin ligands remain to be determined, that data indicate that cell membrane binding determinants conferring high affinity recognition of L-selectin are not strictly conserved: while sulfated glycoprotein ligands may be characteristic of endothelial cells from lymphoid tissues, non-sulfated ligand forms may direct L-selectin-specific cell-cell recognition and adhesion events in other cell types, be it non-endothelial cells or non-lymphoid endothelium.

The above discussion provides a factual basis for the characterization and use of SILL and the method and apparatus for performing adherence assays. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

GENERAL METHODS:

Cell Lines. Cell lines used in these studies were obtained from the following sources: KG1a and Nalm 16, gift of Dr. William E. Janssen; HL60, K562, and Raji, gift of Dr. Lynn Moscinski; COS-7, gift of Dr. Kenneth Zukerman (all from H. Lee Moffitt

Cancer Center, Tampa, FL); RPMI 8402, gift of Dr. Daniel G. Tenen (Harvard Medical School, Boston, MA). All cells were cultured in RPMI 1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified chamber at 37°C with 5% CO_2 in air.

MECA-79 antibody. MECA-79 antibody was a gift from Dr. Phillip Streeter, Searle Research Laboratories/Monsanto Co., St. Louis, MO). A MECA-79 hybridoma is available from American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 as accession number HB-9479. The production of the MECA-79 hybridoma is described in United States Patent 5,403,919.

Preparation of Lymphocytes. Human peripheral blood lymphocytes (PBL) were isolated by Ficoll density gradient from blood drawn in sodium citrate. To obtain rat thoracic duct lymphocytes (TDL), thoracic ducts of rats were cannulated as described by Bollman et al.(1948). Lymph was collected in phosphate buffered saline (PBS) with 0.1% penicillin/streptomycin and 5 U/ml heparin. PBL or TDL were washed three times in RPMI 1640 medium without bicarbonate (Gibco-BRL), pH 7.4, and suspended at 1×10^7 cells/ml in above medium with 5% FBS and kept on ice until use in the adherence assay.

Lymphocyte Adherence Assay. The procedure for the *in vitro* binding of human or rat lymphocytes to KG1a was adapted from the rat lymphocyte-lymph node binding assay which has been described by Stamper and Woodruff (1976) and Sackstein et al. (1988) and described in Example 1. Cytocentrifuge preparations of KG1a or other cell lines were made on a Cytospin 3 Cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA). Frozen rat LN sections 8µm thick were mounted on slides, and lymphocyte binding to LN HEV served as a positive control in all experiments. Slides were air dried, fixed in 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS, rinsed with PBS, incubated in 0.2M L-lysine (Sigma Chemical Company, St. Louis, MO) to block unreacted glutaraldehyde, then rinsed and held in RPMI 1640 with 1% FBS at 4°C until use in experiments.

Lymphocyte suspensions (200 µl) were overlaid onto cytocentrifuge or LN sections in duplicate and placed on a rotating platform (80 rpm) at 4°C for 30 minutes. Slides were then rinsed in cold PBS to remove non-adherent lymphocytes, fixed in 3% glutaraldehyde, and stained with methyl green-thionin. Slides were examined under the light microscope for adherence of lymphocytes to KG1a or LN HEV.

Number of lymphocytes adherent to confluent area of KG1a were counted by light microscopy using an ocular grid under 250× magnification. Quantitation was performed by examining two fields per slide, minimum of two slides per experiment, three separate experiments. Results are presented as % binding compared to corresponding untreated control sections.

Treatment of Lymphocytes with Potential Inhibitors. Lymphocytes in RPMI 1640 medium with 5% FBS were pre-incubated (30 minutes on ice) and the assay performed in the presence of the following inhibitors: 1 mM EDTA (no pre-incubation period); 10 mM D-mannose-6-phosphate (Sigma); 10 µg/ml PPME (kindly provided by Dr. M.E. Slodki, USDA, Peoria, IL); and 5 µg/ml fucoidin (Sigma).

Antibody Blocking Experiments. Lymphocytes (1×10^7 cells/ml) were pre-incubated on ice for twenty minutes with mAbs at 1.0 µg/ml and used in the binding assay without further washing. The following mAbs were used: LAM1-3 (anti-L-selectin) (kind gift of Dr. Thomas Tedder, Duke University, Durham, N.C., and also obtained from Coulter Corp., Hialeah, FL); anti-CD45 (leukocyte Common Antigen) (Becton Dickinson, San Jose, CA); and IgG_1 (isotype control) (Coulter). In some experiments, prepared KG1a slides were incubated with 0.2 µg of anti-CD34 antibodies {HCPAa-1 (clone

My10) and HPCA-2 (clone 8G12) (Becton Dickinson), QBEND10 (AMAC) and 12.8 (kindly provided by Dr. Pat Roth, Coulter Corp.)} in RPMI 1640 with 5% FBS for 30 minutes prior to the binding assay.

PMA Treatment of Lymphocytes. Lymphocytes were suspended at 1×10^7 cells/ml in cell culture medium and incubated 1 hour at 37°C with or without 10 ng/ml PMA (Gibco-BRL). Cells were then washed twice in PBS and used in either the lymphocyte binding assay or analyzed for surface antigens by flow cytometry (see below).

Enzyme Treatment of KG1a or LN.

Cytocentrifuge preparations of KG1a or LN frozen sections were glutaraldehyde-fixed, then treated with various enzymes prior to the binding assay. For treatment with neuraminidase (sialidase), slides were rinsed twice with enzyme buffer (50 mM NaAc, 154 mM NaCl, 9 mM $CaCl_2$, pH 5.5), then incubated 30 minutes at 37°C with 50 µl of buffer (control) or undiluted neuraminidase (1.2 U/ml, Boehringer Mannheim, Indianapolis, IN). In protease studies, slides were incubated with RPMI 1640 alone or RPMI 1640 containing enzymes: 100 U/ml chymotrypsin (Sigma) (115 minutes at 37°C), or 0.1% bromelain (Sigma) (30 minutes at 37°C); to assess specificity, the protease inhibitors PMSF (1.0 mg/ml, Sigma) and chymostatin (900 µg/ml,

Boehringer Mannheim) were coincubated with chymotrypsin (100 U/ml) for 15 minutes at 37°C. Following enzyme treatments, slides were washed three times with RPMI 1640 and placed in RPMI 1640 with 1% FBS until use in the binding assay.

KG1a cells in suspension (4×10^7 cells/ml) were incubated with O-sialoglycoprotein endopeptidase (Accurate Chemical and Scientific Corp., Westbury, NY) (0.24 mg/ml, 37° C, 30 minutes), washed three times with 2% FBS in PBS, and cytocentrifuge preparations were made for use in the binding assay. To verify the activity of the enzyme, cells were tested for the cleavage of CD34 by flow cytometry using QBEND10 mAb.

Antigen Expression by Flow Cytometry. Flow cytometric analysis was performed using the following commercially-available mAbs together with isotype-matched controls: TQ1 (anti-L-selectin), LAM1-3 (Anti-L-selectin), 4B4 (anti-VLA-4) (all from Coulter Corp.); QBEND10 (anti-CD34) (AMAC, Westbrook, ME); anti-CD44, LFA-1-β (anti-CD18), LFA-1-α (anti-CD11a), HPCAS-2 (anti-CD34), anti-CD45, Leukosialin (anti-CD43), anti-Sialyl-Le^x (all from Becton Dickinson). Cells (1×10^6) in 100 µl of PBS with 2% FBS were incubated on ice for 25 minutes with antibody as per manufacturer's recommendations, washed ×3 and analyzed on a FACStar^{PLUS} (Becton Dickinson).

Fluorescence Activated Cell Sorting of KG1a cells. KG1a cells were stained with anti-CD34 mAbs (QBEND10-FITC in two experiments, HPCA-2-PE in one experiment) and positive and negative expressing cells were sorted on a FACstar^{PLUS} flow cytometer equipped with an argon laser tuned at 488 nm (Becton Dickinson). Sorted cell populations were restained with anti-CD34 antibody directed at epitopes not used for sorting and were analyzed to determine the efficiency of the sort. Cytocentrifuge preparations were made of the positive and negative sorted fractions and were used in the lymphocyte binding assay.

Transfection of COS-7 with CD34 cDNA. COS-7 cells were transiently transfected with human full-length CD34 cDNA in pCDM8 plasmid (a gift from Dr. Daniel Tenen, Boston, MA) using a DEAE Dextran transfection method (Selden, 1992). Briefly, COS-7 cells were incubated for 4 hours at 37°C with 10 ml of transfection solution containing 20-40 µg of plasmid DNA, 10% Nu Serum (Collaborative Biomedical Products, Bedford, MA), 400 µg/ml DEAE Dextran (Sigma), and 100 µ chloroquine (Sigma) in Dulbecco's Modified Eagles Medium (Gibcon-BRL). Cells were then rinsed and treated with 10% DMSO (Sigma) in PBS for two minutes at room temperature, rinsed in PBS, and incubated in

tissue culture media for three days. In one set of experiments, trypsinization was avoided by growing transfected cells directly on glass slides for subsequent use in the binding assay or for analysis of CD34 expression by fluorescence microscopy. In other experiments, COS-7 cells grown on 10 cm plates were removed with trypsin/EDTA (0.25%/1 mM, Gibco-BRL), then analyzed for CD34 expression by flow cytometry. These trypsinized cells were then placed on slides by cytocentrifuge for use in the lymphocyte binding assay.

Flow Cytometric Analysis of KG1a and Immunoprecipitation of ^{35}S-metabolically radiolabeled KG1a. Indirect immunofluorescence staining was performed by incubating 1×10^6 KG1a with 1 µg MECA-79 or IgM control antibody (both gifts from Dr. Phillip Streeter, Searle Research Laboratories/Monsanto Co., St. Louis, MO) in 2% FBS in PBS for 30 minutes on ice. After washing, the cells were treated with 5 µg FITC-conjugated secondary antibody for 30 minutes on ice. Flow cytometric analysis was performed on a Facscan flow cytometer (Becton-Dickinson). KG1a were metabolically radiolabeled by incubating 100×10^6 cells (20×10^6 cells/ml) for five hours in CRCM30 sulfate-free medium (Sigma Chemical Co., St. Louis, MO) supplemented with 150 µCi/ml ^{35}S-Na_2SO_4 (Dupont/New

England Nuclear, Boston, MA). Mesenteric lymph nodes from three mice (~60 mg of tissue) were minced and radiolabeled in parallel with the KG1a for five hours in CRCM30 sulfate-free medium supplemented with 1mCi/mi ^{35}S-Na_2SO_4. KG1a and murine lymph nodes were lysed in 2% Triton X-100 in Tris-buffered saline (TBS) containing 1 mM PMSF and 1 µg/ml each of aprotinin, leupeptin and pepstatin A overnight at 4°C. The lysates were clarified by centrifugation at 10,000g for fifteen minutes and the samples precleared with protein G-agarose for four hours. Immunoprecipitation was performed using equivalent amounts of TCA-precipitable counts from lymph node and KG1a lysates, using 2 µg IgM control antibody or MECA79 followed by 6 µg goat anti-rat IgM secondary antibody (Jackson ImmunoResearch Labs Inc.), or 1 µg anti-CD43 or IgG_1, followed by protein G agarose overnight incubation at 4°C. Samples were electrophoresed (7.5% SDS-PAGE) under reduced conditions followed by autoradiography as described previously (Sackstein et al., 1995).

Chlorate Inhibition of Sulfation. The KG1a adherence assay was performed and quantitated as described previously (Oxley and Sackstein, 1994b). The anti-CD43 antibody L60 (Becton-Dickinson) recognizes a sialylated epitope, thus allowing an independent assessment of the efficiency of

neuraminidase treatment of KG1a. KG1a were treated with 0.1 U/ml neuraminidase (*V. cholerae*, Boehringer Mannheim, Indianapolis, IN) for one hour in RPMI 1640 without bicarbonate (Gibco/BRL, Gaithersburg, MD) for sixty minutes at 37°C. Control cells were incubated in RPMI 1640 without bicarbonate with an equivalent volume of neuraminidase storage buffer. The cells were washed in RPMI 1640 twice, tested by the adherence assay to verify complete loss ligand activity, and by flow cytometry to verify loss of the L60 epitope. The neuraminidase-treated KG1a were recultured in RPMI 1640 containing 10% FBS (2×10^6 cells/ml) in the presence or absence of 10 mM sodium chlorate (Sigma Chemical Co.) for twenty-four hours. To verify chlorate inhibition of sulfation, CD43 was immunoprecipitated from equivalent numbers of untreated and chlorate-treated KG1a that were ^{35}S-Na_2SO_4 radiolabeled for the last six hours of the twenty-four hour chlorate treatment.

EXAMPLE 1

The procedure for the *in vitro* binding of human or rat lymphocytes to KG1a cells was adapted from the rat lymphocyte - lymph node (frozen section) binding assay which has been described by Stamper and Woodruff (1976) and Sackstein et al. (1988) as

described herein above. Cytocentrifuge preparations of KG1a or other cell lines were made on a Cytospin 3 Cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA) following the manufacturer's instruction (Cytospin®3 Cell Preparation System Operator Guide) with modifications of the sample chamber to provide appropriate placement of the cytocentrifuge cell pellet onto slides for use in an adherence assay as described herein above.

Cell Separation: Cell separation procedures for blood cells as are generally known to those skilled in the art are used to separate lymphocytes and granulocytes from peripheral blood samples, from bone marrow aspirates, and from vertebral bodies donated through organ harvests. More specifically:

Peripheral Blood

(1) Sodium citrated blue top tubes are used, spun approximately 30 seconds at 100g to pull all cells away from the top of the tubes. The blood is removed, 25 ml or less, and placed into a 50 ml Falcon® tube. Using sterile 1M PBS, add equal volume to the volume of blood for a 1:1 final dilution.

(2) Place 10 ml of FICOLL lymphocyte separation medium into a fresh 50 ml tube. Layer the blood from step 1 onto the top of the FICOLL medium slowly and carefully to insure the interface is tight. Place no more than 35 ml of diluted blood on top of

the FICOLL layer so that the total volume inside the tube does not exceed 45 ml. Spin at 400g for 30 minutes.

(3) Remove the upper clear layer containing the plasma and platelets; remove the next layer (cloudy, lymphocyte layer, PBL: peripheral blood lymphocytes), and place in a 50 ml tube. Suspend the PBL to 45 ml in 1× PBS. Spin them again for 10 minutes at slightly less than 200g (any more force than this will cause platelet contamination). Count cells, wash and spin at 1000 rpm twice and resuspend at 3 to 4 million cells per ml which is the volume necessary to achieve correct cell density in the cytocentrifuge procedure.

(4) Remove the clear FICOLL layer from the tubes from step (3) and discard leaving only the granulocytes and monocytes in the red cell pellet. Dilute the pellet 1:1 by volume with 1× Hank's Balanced Salt Solution (HBSS). Dilute this volume 1:1 with 3% Dextran (made by dissolving 1.5g of Dextran powder into 50 ml of sterile 1× PBS) and shake gently to mix. Allow to stand for 25 minutes so that the red blood cells pellet out under gravity, leaving the granulocytes and monocytes in the supernatant. After 25 minutes, remove the supernatant and dilute it to 45 ml in 1× PBS. Discard the red cell pellet. Spin the

granulocytes and monocytes again for 10 minutes at 200g. To remove remaining red cells treat pellet with a salt gradient. Count the cells and wash one more time at 200g for 10 minutes before resuspending to 3 to 4 million cells per ml. Both groups of cells (PBL and granulocytes) can be held on ice until ready for cytocentrifuge.

Sub B2

Bone Marrow Aspirates: Bone marrow aspirates should be requested in liquid-heparinized tubes. The tube should be well shaken to make sure any cells adhering to bone and tissue are released into the liquid portion of the sample. The tube should then be placed into a 15 ml Falcon® tube and spun for 10 minutes at 1200 rpm. Remove the top layer of bone and tissue from the surface and discard. The remaining aspirate is diluted in 1× PBS and treated as if it ~~were a peripheral blood sample in the above procedure.~~

Though the aspirate material is not supposed to contain red blood cells, FICOLL preparation is still useful for removing bone and tissue which was not removed in the first wash. The cells removed from above the FICOLL layer should be labeled "bone marrow cells" and should be prepared for cytospin in the same manner and to the same concentration as were the PBLSs.

Vertebral Bodies: When working with the vertebral bodies, it is extremely important to

remember the danger associated with working with patient blood. Due to the nature of the procedure used to retrieve the desired cell populations, special precautions must be taken to prevent contact with the fluids and tissues of the sample by the researcher. A full frontal covering must be worn along with gloves and a face guard.

(1) Cut the vertebral body in half and remove the marrow and placed into a solution of heparin. It is better to remove many very small pieces of marrow instead of just a few large chunks. This allows for more surface area of marrow to be exposed, and therefore, more cells can be collected from the bone marrow matrix.

(2) Once all of the marrow has been removed, shake the tube containing the heparinized marrow vigorously, in order to free any cells attached to the matrix. Let the tube stand for a few seconds to allow all of the dense marrow material to sink to the bottom of the tube. Remove the supernatant solution and place it into a clean tube. Resuspend the chunks of marrow in heparin again and shake vigorously to free more cells. Again, remove the supernatant. Repeat several times. Once the marrow is cleansed of most of the adherent cells, spin the supernatant materials in the centrifuge for 10 minutes at 200g. Resuspend the pellet in RPMI medium and pass

the suspension through a sterile nylon mesh filter into a clean 50 ml tube. This will remove any remaining marrow pieces. The remaining cells should be counted and washed two more times before being suspended to proper concentration (3 to 4 million per ml) in RPMI medium for cytocentrifuge.

After each of the cell populations has been separated, washed, and suspended to proper concentration, the following procedure is used to place the cells onto slides in order to test the binding capacity of each population. The cytocentrifuge will pull cells out of small volumes of solution and place them one cell layer thick onto slides. If the concentration of cells in solution is too high, cells will be stacked on top of one another and the experiment will not produce useful results. If the concentration is too low, the actual numerical data collected from the experiment will not be statistically valuable. Cell suspensions should have a concentration of only 3 to 4 million cells.

Cytocentrifuge Procedure

(1) As described herein above, place a cell suspension on a slide and place on the rotational table under the experimental conditions of the adherence assay. Observe where cells aggregate and prepare a model slide (Figure 17) showing this

location by which to mark all other slides.

(2) After marking the proper number of slides (i.e. typically one slide/2.5×10^5 cells), take the same number of clamps, funnels, and filter cards (paper guides) (Figure 10) as slides needed. Assemble the holder assembly and load in cytocentrifuge as per the instructions in the cytocentrifuge manual. Once the holder assemblies are loaded, place 100 µl of the proper cell suspension in each funnel. Set speed at 570 and time 5 minutes for this particular assay. As per instruction in manual remove slides from holding member after spin. Do not smear or wipe the delicately placed cells from the slide. Allow slides to dry for one minute.

(3) When dry, place the slides into a 3% glutaraldehyde solution (3.6 ml of 25% glutaraldehyde to 26.4 ml of 1× PBS per 12 slides) for ten minutes. After the 10 minutes of fixing, rinse the slides twice, gently, in 1× PBS. Place the slides into lysine solution (0.2M) for 10 minutes. This solution will prevent nonspecific binding in the adherence assay. After lysine treatment, rinse slides twice in RPMI and then place them into 1% FBS in RPMI at 4°C until ready to perform the adherence assay.

EXAMPLE 2

Lymphocytes Bind to KG1a. Lymphocytes (both PBL and TDL) adhered specifically and reproducibly to KG1a, but not to RPMI 8402, HL60, Nalm 16, K562, or Raji cell lines in the *in vitro* binding assay (Table 1). All experiments were performed in parallel with LN frozen sections as positive controls. Lymphocyte binding to KG1a was observed under conditions identical to those whereby L-selectin mediates binding of lymphocytes to LN HEV.

Lymphocyte Binding to KG1a is Mediated by L-selectin. To directly examine whether lymphocyte attachment was mediated by L-selectin, PBL were pre-incubated with the anti-L-selectin mAb LAM1-3, anti-CD45, or IgG_1 isotype control antibodies. The LAM1-3 antibody completely inhibited lymphocyte binding to KG1a and LN control, while CD45 and isotype control mAbs did not affect binding (Fig. 1A & 1B). In order to quantify the relative amounts of antibody attachment to lymphocytes, antibody-treated lymphocytes were incubated with goat-anti-mouse FITC-conjugated secondary antibody and analyzed by flow cytometry. Although the amount of anti-CD45 antibody on lymphocytes was significantly greater than that of LAM1-3 as indicated by mean channel fluorescence (Fig. 2A-C), LAM1-3 alone blocked lymphocyte adherence to

KG1a and LN HEV, indicating that this effect was specific and not secondary to charge or stearic alterations of the lymphocyte membrane.

The Effect of Enzyme Treatment of KG1a on Lymphocyte Binding. Pretreatment of both KG1a and LN control sections with neuraminidase (60 mU), chymotrypsin (100 U/ml) or bromelain (0.1%) prior to the binding assay abrogated binding of lymphocytes, while treatment with buffer or medium alone did not alter binding capacity. In addition, the effects of chymotrypsin were confirmed by coincubation with the protease inhibitors chymostatin and PMSF, which prevented chymotrypsin effects on lymphocyte binding. However, pretreatment of KG1a with O-sialoglycoprotein endopeptidase had no effect on lymphocyte binding despite complete enzymatic removal of the CD34 epitope recognized by QBEND10 mAb (see Table 2).

Lymphocyte Binding to KG1a is Calcium Dependent. Lymphocyte binding to KG1a and to LN control sections was completely inhibited by the presence of EDTA, indicating a calcium requirement for lymphocyte-KG1a binding.

Mannose-6-Phosphate, PPME, and Fucoidin Inhibit Lymphocyte Binding to KG1a. The specificity of lymphocyte-KG1a binding was investigated by treating PBL or TDL with carbohydrate inhibitors of L-

selectin-HEV interactions prior to the adherence assay. Man-6-P (10 mM), PPME (10 μg/ml), and fucoidin (5 μg/ml) all inhibited lymphocyte binding to both KG1a and LN control sections (see Table 2).

PMA Treatment of Lymphocytes Results in the Loss of Binding to KG1a. PBL were incubated for 1 hour at 37°C with 10 ng/ml PMA, then used in the lymphocyte binding assay. PMA-treated PBL were unable to bind to either KG1a or LN HEV, while control PBL demonstrated high amounts of binding (see Table 2).

Loss of surface L-selectin was assessed by flow cytometric analysis of TQ1 levels in control and PMA-treated PBL. PMA-treated lymphocytes showed a dramatic decrease in TQ1 mean channel fluorescence (to levels less than 10% of that of untreated cells) in three separate experiments. PMA-treated PBL were also analyzed for expression of CD44, LFA-1 (both α and β chains), and VLA-4, and expression of these adhesion molecules following PMA exposure was identical to expression on control PBL.

EXAMPLE 3

Pretreatment of KG1a with Anti-CD34 Antibodies Did Not Inhibit Adherence of Lymphocytes. Cytocentrifuge preparations of KG1a were preincubated

with anti-CD34 antibodies and the binding assay was performed in the presence of the antibodies (Table 2). Monoclonal antibodies to four different CD34 epitopes were used alone or in combination, including the clones My10, QBEND10, 8g12, and 12.8, in amounts ranging from 0.2 to 17 µg/slide. Anti-CD45 (irrelevant control) and IgG_1 (isotype control) antibodies were also tested. None of the anti-CD34 antibodies inhibited lymphocyte binding to KG1a, despite immunohistochemical evidence of extensive antibody binding to the glutaraldehyde-fixed KG1a sections.

Other Surface Antigens on KG1a do not Appear to Mediate Binding. The surface expression of several antigens on KG1a, RPMI 8402, HL60, Nalm 16, K562, and Raji was analyzed by flow cytometry (Table 1). LFA-1, FLA-4, CD44, Sialyl Le^x, and CD43 were all expressed by KG1a and at least one other cell line that did not support lymphocyte adherence. Of note, although RPMI 8402 cells express CD34 at levels comparable to KG1a, there was no adherence of lymphocytes to these cells in the binding assay.

CD34 Positive and Negative KG1a Cells Supported Equivalent Amounts of Lymphocyte Binding. CD34+ and CD34- KG1a cells were separated by fluorescence activated cell sorting and cytocentrifuge preparations of each population were made. The *in*

vitro adherence of lymphocytes was identical in the CD34+ and CD34- populations despite an enrichment of >90% and <10% CD34+ cells in the respective populations (Figs. 3A-D and 4; Table 2).

EXAMPLE 4

CD34-Transfected COS-7 Cells Did Not Support Lymphocyte Adherence. COS-7 cells were transfected with CD34 and tested in the *in vitro* binding assay, and both trypsinized and intact transfected COS-7 cells failed to support lymphocyte adherence. By flow cytometric analysis, transfected cells were approximately 60% positive for CD34 expression, and the mean channel fluorescence was greater than that of KG1a control cells (Fig. 5A-F). Intact, untrypsinized COS-7 cells transfected with CD34 also strongly expressed CD34 (≈90% positive as estimated by fluorescence microscopy).

EXAMPLE 5

Flow cytometric analysis of KG1a and immunoprecipitation of ^{35}S-metabolically radiolabeled KG1a demonstrate an absence of MECA79 antigen.

In functional studies, incubation of KG1a cytocentrifuge layers with MECA 79 or isotype-control

(IgM) rat monoclonal did not inhibit lymphocyte attachment in the adherence assay (e.g., for studies using MECA 79 at a concentration of 50 µg/ml, binding was 98.2% ± 3.38% (mean ± SEM) that of control assays without antibody addition). However, as is characteristic of MECA 79, lymphocyte attachment to murine lymph node HEV was blocked using MECA 79 at concentrations as low as 2 µg/ml. To investigate surface expression of MECA 79 antigen on KG1a cells, flow cytometric analysis of MECA-79 antigen was performed on KG1a cells and compared with isotype matched control antibody (Figs. 6A and 6B). Although lymph node HEV were clearly and characteristically identified by staining with MECA 79 as evident by fluorescence microscopy. There was no MECA 79 staining evident by flow cytometric analysis of KG1a cells by fluorescence microscopy.

To further evaluate for expression of MECA 79 antigens by KG1a, the cells were incubated with either ^{35}S-Na_2SO_4 or ^{35}S-methionine/cysteine and resultant radiolabeled protein was immunoprecipitated using MECA 79 antibody (Figure 6C). As a positive control, murine lymph nodes were radiolabeled, lysed, and immunoprecipitated in parallel with KG1a. To adjust for potential differences in efficiency of radioactive label incorporation between KG1a and murine lymph nodes, the relative amounts of lysate

immunoprecipitated from each source were equalized for TCA-precipitable counts. A representative immunoprecipitation of MECA 79 from ^{35}S-Na_2SO_4-labeled KGLa and lymph node is shown in Figure 6C. The characteristic 50,000 and 90,000 mw bands are immunoprecipitated by MECA 79 in lymph node lysate but no MECA 79 bands are detected in the KGla lysate; similar results were obtained with MECA 79 immunoprecipitation of ^{35}S-methionine/cysteine labeled KG1a lysates. In the figure, the faint band at ~90,000 mw shown on both MECA 79 and rat IgM-control antibody immunoprecipitates of KG1a cells is non-specific as it can be removed by extensive preclearing of lysates with protein-G agarose alone; a similar faint 90,000 mw band could also be visualized on IgG1 control immunoprecipitation of $^{35}SO_4$-labeled KG1a cells. Of note, the sulfated glycoprotein CD43 (~110 kDa) was readily immunoprecipitated from KG1a lysates, verifying that $^{35}SO_4$ was incorporated into newly synthesized products and that immunoprecipitation conditions were appropriate for detection of specific radiolabeled KG1a proteins.

EXAMPLE 6

Chlorate inhibition of sulfation does not block lymphocyte binding to KG1a. As shown herein

above, the KG1a L-selectin ligand, like the endothelial ligands, requires sialylation for function, as treatment of KG1a with neuraminidase completely abolishes L-selectin-mediated lymphocyte binding. Applicant, therefore, measured the kinetics of recovery of ligand activity following neuraminidase treatment, and chose experimental conditions such to maintain chlorate inhibition of sulfation throughout the period when the KG1a ligand is being re-expressed on the cell membrane. Cell viability following neuraminidase digestion was typically >99% by trypan exclusion.

Initial studies of the kinetics of recovery of binding activity following neuraminidase treatment showed absence of ligand activity for 10-12 hours; thereafter, ligand activity increased steadily with return to baseline levels within 24 hours. Return of binding activity following desialylation was blocked by metabolic inhibition of N-linked glycosylation by tunicamycin and by inhibition of protein synthesis by cycloheximide (Table 3). Of interest, the results of tunicamycin and cycloheximide experiments indicate that the membrane turnover of the L-selectin ligand is relatively slow in resting cells, as culture of KG1a in the presence of these reagents for up to 20 hours did not alter L-selectin ligand activity (Table 3). However, following neuraminidase

treatment of KG1a, both reagents inhibited return of ligand activity without affecting cell viability (trypan blue exclusion >98%), suggesting that re-expression of ligand activity results from de novo synthesis and post-translational processing of ligand and not from transport of pre-formed ligand from intracellular compartments to the membrane surface.

The cleavage of sensitive sialic acid epitopes following neuraminidase treatment of KG1a was confirmed by testing for loss of ligand activity in the adherence assay and by measuring the expression of the sialic acid-dependent L60 epitope of CD43 by flow cytometry (Table 3; Figures 7 and 8). L60-negative/ ligand activity-negative neuraminidase-treated KG1a were incubated without chlorate (control) or in presence of chlorate for 24 hours to inhibit sulfation throughout the duration of ligand reexpression. Cell viability was >95% in both chlorate-treated and control cell populations (trypan blue exclusion). Twenty-four hours of chlorate treatment (Figure 7C) did not alter the re-expression of ligand activity; indeed, quantitation of adherence assays performed on KG1a 24 hours after neuraminidase treatment revealed that L-selectin ligand activity in chlorate-incubated KG1a cells was slightly higher than that of non-incubated control cells (Table 3). Quantitated lymphocyte adherence to chlorate-incubated KG1a cells

was 107% +/- 6% (mean +/-SEM) that of neuraminidase-treated KG1a cells not incubated in chlorate. As with control KG1a cells, lymphocyte adherence to sulfate-treated KG1a was L-selectin-specific: it was Ca++-dependent, was completely inhibited by anti-L-selectin monoclonal antibodies known to block L-selectin adhesive function and by carbohydrate molecules known to bind L-selectin (e.g., fucoidin), and eliminated by phorbol myristate acetate (PMA) treatment of lymphocytes to induce L-selectin shedding.

Sequential ^{35}S-SO_4-pulse radiolabelling studies at eight hour time intervals within the 24 hours of chlorate incubation indicated that sulfation was inhibited throughout the entire incubation period, as demonstrated by diminished quantities of TCA-precipitable radiolabeled protein counts (chlorate-incubated counts consistently <10% that of non-chlorate treated controls) and of total $^{35}SO_4$-radiolabelled proteins observed by SDS-PAGE/autoradiography of cell lysates (Figure 9A,B). However, chlorate did not inhibit total protein synthesis as ^{35}S-methionine/cysteine incorporated TCA-precipitable counts were not significantly different in chlorate and control groups, ^{35}S-SO_4-radiolabelling of CD43 was markedly diminished by chlorate treatment, even in the terminal eight hours of the 24 hour incubation. Similar to the results of recovery of

ligand activity, chlorate treatment did not block re-expression of CD43 to baseline levels (Figure 8C) despite evident sulfation-deficiency (Figure 9). Monitoring of membrane CD43 recovery was performed using a sialylated epitope of the protein, indicating that sialylation of CD43 (like sialylation of the L-selectin ligand) was not affected by chlorate treatment.

Flow cytometric analysis of CD43 shown in Figure 8 demonstrated a loss of a sialylation-specific epitope following neuraminidase treatment (Fig. 8B, bottom) relative to control levels (Fig. 8A, top).

Inhibition of sulfation by sodium chlorate is shown in Figure 9 demonstrated by immunoprecipitation/SDS-PAGE analysis of ^{35}S-SO_4 incorporation into CD43. Numbers at the left of the figure are molecular weights in kDa.

EXAMPLE 7

PSGL-1 Analysis

P-selectin glycoprotein ligand-1 (PSGL-1) is a cell surface mucin-like glycoprotein which serves as a ligand for both P- and E-selectin. This protein is expressed on a variety of myeloid cell lines, including HL60 cells. In order to determine whether the L-selectin ligand activity of KG1a is related to PSGL-1, Applicants performed flow cytometric analysis

of PSGL-1 expression on KG1a, blocking studies utilizing both a P-selectin-Ig chimera and an anti-PSGL-1 monoclonal antibody, and enzymatic cleavage and metabolic studies of PSGL-1 and L-selectin ligand activity. These studies reveal that KG1a cells express immunoreactive and functional PSGL-1, however, expression of PSGL-1 is separable from L-selectin ligand activity: (1) blocking of PSGL-1 function using P-selectin-Ig chimera and anti-PSGL-1 monoclonal antibody had no effect on ligand activity of KG1a cells; (2) cleavage with the proteases mocarhagin and O-sialoglycoprotease each abrogated PSGL-1 recognition by P-selectin-Ig chimera and L-selectin activity of PSGL-1, but had no effect on L-selectin ligand activity; (3) metabolic inhibition of sulfation by chlorate eliminated P-selectin ligand activity of PSGL-1 but did not alter L-selectin ligand activity; and (4) KG1a sorted for high-level (>95%) and low-level (>5%) expression of PSGL-1 had equivalently high L-selectin ligand activity, and, moreover, HL60 cells were completely devoid of L-selectin ligand activity despite high PSGL-1 expression. These studies indicate that although PSGL-1 is capable of serving as a ligand for E- and P-selectin, it does not itself, display L-selectin ligand activity. The independent relationship between PSGL-1 and L-selectin ligand activity indicates these functions are subserved by

distinct molecules.

Throughout this application various publications or patents are referenced by citation or number. Full citations for the publications referenced are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE

Expression of Surface Molecules on Cell Lines Utilized in the Lymphocyte Adherence Assay.

CELL LINE	LYMPHOCYTE ADHERENCE	LINEAGE	RELATIVE EXPRESSION OF MEMBRANE PROTEINS*					
			CD34	LFA-1	VLA-4	CD44	Sialyl Le^x	CD43
KG1a	YES	Myeloid	++++	++++	++++	++++	++++	++++
RPMI 8402	NO	Lymphoid	++++	-	++++	+++	-	++++
HL60	NO	Myeloid	-	-	++++	+	-	++++
Nalm 16	NO	Lymphoid	-	-	++++	-	+++	++++
K562	NO	Erythroid	-	-	-	-	++	+++
Raji	NO	Lymphoid	-	+++	++++	-	-	+

*Percentage of positive cells as determined by flow cytometric analysis.
- = 0-5% positive
+ = 6-35% postive
++ = 36-65% positive
+++ = 66-95% positive
++++ = >96% positive

Table 2. Lymphocyte Adherence to KG1a

LYMPHOCYTE TREATMENT	Mean (SEEM) of Binding (% of Untreated Control)
EDTA	0.3 (0.3)
Mannose-6-P	5.7 (1.0)
Fucoidin	1.4 (0.4)
PPME	5.4 (0.5)
LAM1-3 mAb	1.9 (0.4)
Anti CD45 mAb	98.7 (6.3)
IgG_1 Control mAb	115.1 (9.0)
PMA	1.1 (0.3)
KG1A TREATMENT:	
Anti CD34 mABs†	116.2 (7.7)
Anti CD45 mAb	98.0 (3.6)
IgG_1 Control mAb	101.8 (8.5)
CD34-Positive Sort	102.8 (3.5)
CD34-Negative Sort	104.1 (4.2)
Neuraminidase	3.1 (0.7)
Neuraminidase Buffer Control	100.5 (6.7)
O-Sialoglycoprotein Endopeptidase	98.4 (2.3)
Bromelain	3.8 (0.4)
Chymotrypsin	6.7 (0.7)
Chymotrypsin, PMSF, Chymostatin	94.0 (3.8)

Combination of HPCA-1, HPCA-2, 12.8 and QBEND10 mAbs.

TABLE 3

Lymphocyte Adherence to KG1a

KG1a Treatment	Mean (SEM) of Binding (% of untreated control)**
Neuraminidase (t=0, no culture)	0.02 (0.1)
Neuraminidase Buffer Control (no culture)	98.5 (7.5)
Neuraminidase, 24 hr culture	101.3 (6.5)
Neuraminidase, 24 hr culture with Chlorate (10 mM)	107.1 (6.2)
Neuraminidase, 20 hr culture with Tunicamycin (15 ug/ml)	0.4 (0.1)
Neuraminidase, 20 hr culture with Tunicamycin Buffer alone	105.2 (7.5)
20 hr culture with Tunicamycin (15 ug/ml) (no neuraminidase pretreatment)	106.2 (7.2)
Neuraminidase, 20 hr culture with Cycloheximide (1.25 ug/ml)	0.5 (0.2)
20 hr culture with Cycloheximide (1.25 ug/ml) (no neuraminidase pretreatment)	91.8 (5.8)

*Following neuraminidase treatment, all cells were washed prior to culturing as indicated. Experimental details are described in text.
**Number of lymphocytes adherent to confluent area of KG1a were counted by light microscopy using an ocular grid under 250X magnification (quantified a minimum of 2 fields/slide, 2 slides/experiment, 3 separate experiments). Results are presented as percent binding compared with corresponding untreated control KG1a cyto-spin preparations.

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